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THE MECHANISM OF THE SHADOW REFLEX IN CIRRIPEDIA III. RHYTHMICAL PATTERNED ACTIVITY IN CENTRAL NEURONS AND ITS MODULATION BY SHADOWS [1]

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One of the most noteworthy characteristics of behavior in the Cirripedia is the rhythmical nature of the so-called "fishing" operation. This consists of the extension of the body between the opercular plates, the extension of the cirri to form a net, followed by rolling up of the cirri and retraction back into the shell. The frequency of the fishing event is variable and depends in part on the species, size, temperature, oxygen concentration, pH, etc. (Southward, 1955a, 1955b, 1957, 1962, 1964; Crisp and Southward, 1961; Southward and Crisp, 1965). Some barnacles (*e.g.*, the stalked intertidal barnacle, *Pollicipes* (=*Mitella*)) do not engage in rhythmical fishing activity but simply extend the cirral net into a moving stream of water that is provided by currents in the habitat (Barnes and Reese, 1960). In addition to fishing, various degrees of "pumping" may be exhibited by barnacles. This consists of activity ranging from incomplete body extension and retraction to opening and closing the opercular plates. Blatchford (1970) has reported on another repetitive contractile process that subserves the function of circulating blood and thus substitutes for the contractile portion of the circulatory system that is lacking in Cirripedia.

Each of these activities is repeated more or less regularly within a characteristic frequency range. Such activity, of course, requires a set of controlling neural events, and Gwilliam and Bradbury (1971) reported on the occurrence of rhythmical patterned bursts of activity recorded from various nerve trunks in the isolated central nervous system of *Balanus cariosus* (Pallas). At that time an argument was presented to the effect that such patterned activity was centrally generated, and that this neural activity constituted a program that initiated and sustained the normal rhythmical behavior of the barnacle.

Another important aspect of barnacle behavior that has been recently investigated is the shadow reflex (Gwilliam, 1963, 1965; Millecchia and Gwilliam, 1972). The shadow reflex is a series of events beginning with the detection of a sudden decrease in light intensity. Following this, a sequence of neuronal events ultimately leads to the interruption of on-going activity in the barnacle, the withdrawal of the body into the protective shell, and the closure of the opercular plates. This withdrawal-closure response must involve both excitatory and inhibitory mechanisms. Upon withdrawal the nerves responsible for the contraction of muscles that cause extension must be turned off; those accomplishing withdrawal would have to be activated at the same time. Since there is no evidence of peripheral inhibition in barnacles (Hoyle and Smyth, 1963), it is likely that effects of central

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inhibition will be seen in the activity of motor neurons. The shadow reflex, then, may be viewed as the series of events leading to withdrawal-closure, and a simple model for the initial stages has been proposed (Millecchia and Gwilliam, 1972; Lantz and Millecchia, 1975).

The above considerations suggest that it should be possible to locate central neurons that have rhythmical patterned outputs; that there should be at least two major categories of such neurons that are out of phase (see Gwilliam and Bradbury, 1971), and it should be possible to identify each group of neurons with particular muscles that are involved in extension and in withdrawal-closure. Further, the extension-related neurons should show inhibition at "light-off" while the withdrawal-related neurons should show excitation.

As reported in Gwilliam and Bradbury (1971) and previously (Gwilliam, 1968), it has proved possible to penetrate and hold a variety of cells in the ventral ganglion of the barnacle, and certain of these cells show the same activity patterns as are seen in the nerve trunks. Using such recordings as the basic approach, it has been possible to explore some of the relationships between individual cells, the motor output seen in nerve trunks, and in some cases the activity of muscles. So far it has not been possible to utilize all of the "*Tritonia* technology" introduced by Willows (1967), but the ultimate aim of this work is similar—to explain behavioral events in terms of nerve cell activity patterns and interactions.

This paper reports on the characteristics of certain of these central neurons and on their activities in relation to the behavior of the barnacle. Preliminary reports of some of this material have appeared (Gwilliam, 1973; Gwilliam and Millecchia, 1975).

Materials and Methods

Observations were made using the central nervous systems of *Balanus cariosus* (Pallas) from the Oregon coast, and general techniques were worked out on that species. This was followed by work with preparations from *Balanus hameri* (Ascanius) dredged from about 500 yards south of Langness, Isle of Man, in 10–15 fathoms. Later, more detailed observations and experiments were performed on *B. cariosus*.

Dissections were done as described previously (Gwilliam, 1965; Gwilliam and Bradbury, 1971). Three types of preparations were used in this study: the isolated central nervous system (Gwilliam and Bradbury, 1971, Fig. 1); the CNS with the last three pairs of cirri attached; and the CNS with cirri and the opercular plates with the adductor muscle intact (Fig. 1). All preparations included the median photoreceptor. Preparations were immersed in either "Instant Ocean" artificial sea water, a simpler artificial sea water, or natural sea water (the latter only at the Menai Bridge Marine Sciences Laboratory), cooled to 11°–14° C, by means of a submerged coil of polyethlene tubing through which cold water was pumped.

A bathing medium containing an excess of Mg^{2+} and a reduced amount of Ca^{2+} was used to block synaptic transmission and had the following composition: NaCl, 327 mM; KCl, 9.7 mM; $CaCl_2$, 1.0 mM (*ca* 0.1 ×); $MgCl_2$, 125.0 mM (*ca* 2.5 ×); Na_2SO_4, 28.2 mM; TRIS, 2.0 mM. This was later modified to contain only 75 mM $MgCl_2$ (*ca* 1.5 ×) with an appropriate adjustment in NaCl to maintain the same osmolarity.

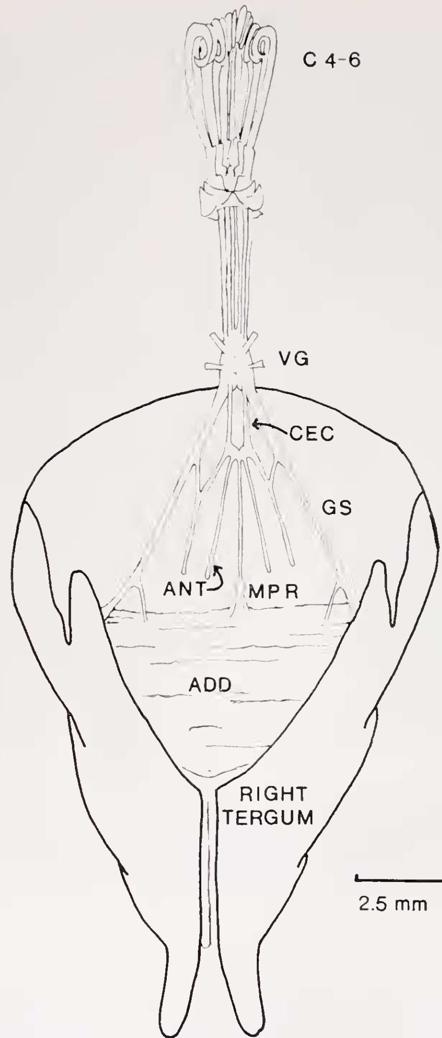

FIGURE 1. Diagram of the Cirral-CNS-Adductor Muscle preparation from *Balanus cariosus*. ANT represents antennular nerve; ADD, adductor muscle; C4-6, cirri 4 through 6; CEC, circumesophageal connective; GS, great splanchnic nerve; MPR, median photoreceptor; VG, ventral ganglion.

In view of the fact that this solution had a reduced amount of Na^+ a control was run using a medium with the normal amount of Mg^{2+} and Ca^{2+}, but with low Na^+. This solution had the following composition: NaCl, 326 mM; KCl, 9.7 mM; $CaCl_2$, 13.3 mM; $MgCl_2$, 49.0 mM; Na_2SO_4, 28.2 mM; TRIS, 97 mM. In addition controls were run with simple artificial sea water with the following composition: NaCl, 423.0 mM; KCl, 9.7 mM; $CaCl_2$, 13.3 mM; $MgCl_2$, 49.0 mM; Na_2SO_4, 28.2 mM; TRIS, 1.0 mM. The pH of these solutions was 7.6–7.8.

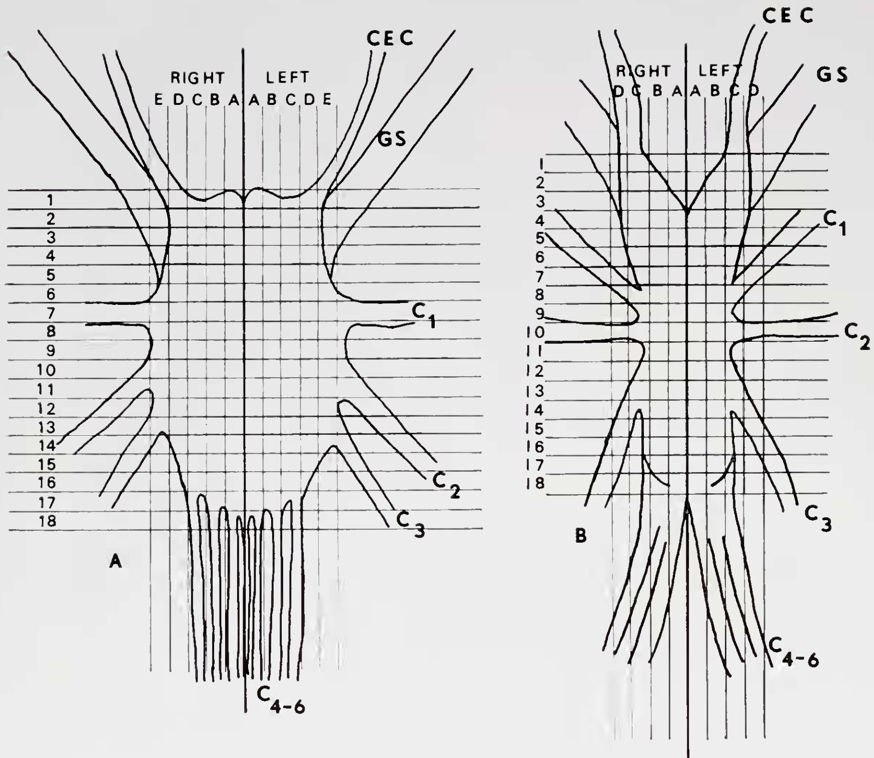

FIGURE 2. Grid reference system applied to diagram of ventral view of the ventral ganglion of each of the species of barnacles used: A, *Balanus cariosus*; B, *Balanus hameri*. The notation system designates the letter corresponding to the lateral position of the unit in question, R or L as a subscript to denote on which side of the midline the unit is located, and a number to place the unit along the anterio-posterior axis. CEC represents circumesophageal connective; C1-6, cirral nerves; GS, great splanchnic nerve.

Recording techniques were conventional, using 2M–3M KCl-filled glass micropipette electrodes of over 25 Megohms DC resistance (measured in sea water). A seawater bridge to a calomel-mercury half-cell or a Ag-AgCl pellet served as an indifferent electrode, and a similar cell was constructed to serve as the electrode holder. Neutralized input capacity amplifiers were used for intracellular recording, and one of these was designed to permit simultaneous stimulation and recording (W. P. Instruments, Camden, Conn.). External recording was accomplished with Ag–AgCl or Pt. iridium hook electrodes, or with suction electrodes. Externally recorded activity was AC amplified, and all recordings displayed on a cathode ray oscilloscope and photographed in the usual manner. Occasionally records were made on an ink-writing oscillograph.

After some experience it became apparent that certain types of cells were consistently found in the same relative positions in the ganglion. When the ganglion is viewed with transmitted light delivered *via* an image conduit embedded in the wax floor of the recording chamber, it is possible to see many neuron somata on

the ventral surface and penetrate them with an electrode under visual control. A simple grid system was devised (Fig. 2) that permitted describing the positions of the recording site relative to other landmarks. In this way it soon became apparent that somata located in particular regions had particular properties, but they were not otherwise identifiable. In a few cases certain somata are identifiable from preparation to preparation because of their size and consistent location. Unfortunately, the role of these neurons in the behavior of the animals remains unknown. Obviously, this technique does not insure soma recordings, but it renders them more likely. As will be apparent below, it is possible that some recordings were from the neuropile, but the attempt was always made to restrict the depth of penetration of the electrode to the surface cells.

Penetration of cells in the intact ganglion proved virtually impossible. It was therefore necessary either to desheath the ganglion or to treat it with pronase. Both techniques proved satisfactory under most conditions. Carefully controlled application of pronase, 1–10 mg/ml, for 1.0–4.0 minutes at 10°–13° C proved to be quite reliable. On those occasions when it was desired to position two electrodes in the ganglion, desheathing was the method of choice. It was usually necessary to tap the manipulator to penetrate cells in pronase-treated ganglia, and doing this for the second cell almost always dislodged the electrode from the first cell. At irregular intervals selected nerve trunks were monitored with suction electrodes during pronase treatment and before and after desheathing to assess the effect of these procedures. There was no consistent permanent alteration of output detectable 30 minutes after the operations had been completed.

Stimulation was accomplished *via* hook or suction electrodes on one or more nerve trunks or by passing current through an intracellular electrode. A simple switching arrangement made it possible either to record from or stimulate a given nerve trunk *via* its suction electrode.

A few serial sections of the ventral ganglion of *B. cariosus* were prepared to aid in localizing neuron somata. Procion yellow and cobalt acetate injection studies have been undertaken to support the localization information gained in other ways.

Results

Activity

A review of barnacle functional morphology may facilitate understanding of some of the matters discussed below The following information is derived from unpublished observations, from Gutmann (1960), Crisp and Southward (1961), Bullock and Horridge (1965), and Gwilliam and Bradbury (1971).

The nervous system. The central nervous system of balanoid cirripeds consists of a small, bilobed supra-esophageal ganglion, connected to the ventral ganglion *via* the circumesophageal connectives. The ventral ganglion is composed of the fusion of several primitively separated segmental ganglia (seen in, *e.g., Mitella*) into one mass that shows little superficial trace of segmental structure (Batham, 1944; Cornwall, 1953; Bullock and Horridge, 1965). The topography of the principal nerve trunks is adequately described in various sources, including Gwilliam and Bradbury (1971), but no detailed histological description of the *Balanus* central nervous system has been published to date. Examination of sections of the ventral

ganglion shows nerve cell somata located around the periphery, mainly on the ventral surface, with central neuropile. Cell sizes are variable, ranging from 80 μm to around 5 μm. Cells in locations that correspond to positions of the bursters to be described are mostly in a size range of 20–30 μm, and it is assumed that such is the size range of most of the cells penetrated.

Behavior. The action of various muscles during the main behavioral act of a barnacle—fishing or "beating"—has been described by Gutmann (1960) and by Crisp and Southward (1961). The operculum is connected to the shell by means of a flexible chitinized membrane and can move in all directions. Upward movement is brought about by fluid pressure within the membrane sinus and is limited by the elasticity of the membrane and the degree of relaxation of the three pairs of opercular depressor muscles. The prominent adductor muscle bridges the two scuta, and its contraction closes the valves. According to Crisp and Southward (1961), the adductor is involved in rhythmical activity and does not act like a bivalve mollusc adductor in holding the valves closed when the barnacle is out of water. Hoyle and Smith (1963), however, describe a possible mechanism permitting long term contraction without fatigue in this muscle.

The six pairs of cirri differ in form and function. The first three pairs are short and stout, the other three pairs are long and slender. Basal musculature is similar in all cirri, but the longer cirri have only flexor muscles in the rami which roll them up for withdrawal into the mantle cavity. They are extended by the movement of body fluid forced into them by muscles in the prosoma.

While it is only possible to infer detailed muscle action from the movement (or lack of it) of the body, it seems most probable that opening of the operculum occurs in the following way (Gutmann, 1960): in the closed position, the adductor muscle and the rostral scutal and carinal tergal depressor muscles are contracted. The lateral scutal depressors are relaxed, as are the basal mantle muscles. Under these conditions the operculum tends to be rather flat. When opening is taking place, the basal mantle muscles contract, forcing fluid from the base into the subtergal sinus and surrounding tissue; the tergal depressors and the rostral scutal depressors are relaxing, and the effect is to elevate the terga due to the increased pressure in the sub-tergal sinus. As the terga are thrust up, the scuta are carried along, because they are connected to the terga. The adductor muscle relaxes, and the lateral scutal depressors are contracted. This pulls down the lateral edges of the scuta at their widest point and effects opening of the opercular plates. The plates may be opened and shut in this extended condition by antagonistic action of the adductor and the lateral scutal depressors. The sequence would be: a) tergal and rostral scutal depressors relax; b), simultaneously the basal mantle muscles contract; c), the adductor scutorum muscle relaxes; and d), the lateral scutal depressors contract. This sequence gets the operculum open so the body may be extended.

While the animal is closed, all the dorsal musculature, the main longitudinal ventral body musculature, the oral cone depressors, and the adductor are contracted. Just prior to, and continuing coincident with valve opening, paired muscles attached to the scuta and the lateral body exoskeleton (numbered 1, 2, 3, and 8 by Gutmann, 1960) contract as the dorsal and ventral musculature of the body relaxes. This presses the body up into the angle of the valves, causes fluid to move from the

prosoma to the thorax and extends the thorax and the cirri. As the valves open, the body is thrust out through the opening, and the last three pairs of cirri unroll to form the net. Another muscle (designated no. 6 by Gutmann, 1960) elevates the oral cone. Reversal of these actions would result in retraction.

Innervation of muscles. The distribution of nerves to muscles is not well known, but some nerve trunks have been traced with the aid of methylene blue staining and electrical stimulation. These observations have established that the great splanchnic nerve supplies motor fibers to the adductor muscle, the oral retractors, the lateral muscles designated no. 8 by Gutmann (1960), and some other muscles associated with the mantle cavity. The antennular nerve serves both pairs of scutal depressors and probably the tergal depressors. The cirral and paracirral nerves send fibers not only to the cirri but to body musculature. The nerve designated as the mid-dorsal nerve in Gwilliam and Bradbury (1971) has been traced to transverse (circular) muscles dorsal to the nervous system but ventral to the gut. Contraction of these muscles causes increased pressure and movement of body fluid and is probably involved in extension of the body.

Neurophysiology

Figure 2 consists of diagrams of a ventral view of the ganglion of each of the species used with the grid system appropriate to each. The ganglia are morphologically similar, and most generalizations to be reported apply to both species. The shadow reflex in *B. hameri* is not as well developed as it is in *B. cariosus*, but in other respects I am unable to detect major differences. The neurons that have been studied to date are located on the ventral surface and lateral aspects of the ganglia. Those on the dorsal surface have not been systematically examined.

A general search of the ganglion reveals many units that are silent and cannot be driven with application of up to 15 na of depolarizing current. Others are silent but can be driven, still others respond to "light-off" by firing one to many spikes, while others are spontaneously active. The spontaneously active units may be pacemaker-like, irregularly active and obviously receiving considerable synaptic input, or produce quite regular patterned output (Fig. 3). This latter will be referred to as "bursting" and the units called "bursters" because of the similarity of this output to cells so designated in other invertebrates (*e.g.*, Strumwasser, 1965). Units other than the bursters have not yet been linked to any behavioral event other than the coincidence of an "off response" seen in some. The bursters, on the other hand, may be correlated to behavioral events, and a description of their properties and activities forms the main part of this paper.

Identification of types of units. Systematic penetration of large numbers of somata on the ventral surface of the barnacle ventral ganglion has revealed a pattern of distribution of two general categories of bursters: the Midline Inhibited Bursters (MIB) and the Lateral Excited Bursters (LEB). The MIBs are usually found along the midline and are inhibited at light-off (Fig. 3A, B), while the LEBs are usually found laterally and are excited at light-off (Fig. 3C). In *B. hameri* the rather tenuous nature of the shadow reflex in the isolated CNS forces a greater reliance on position of the unit for identification, but other characteristics of the bursting patterns usually support the positional recognition. LEBs in general have shorter bursts and shorter interspike intervals (higher frequency) earlier in the

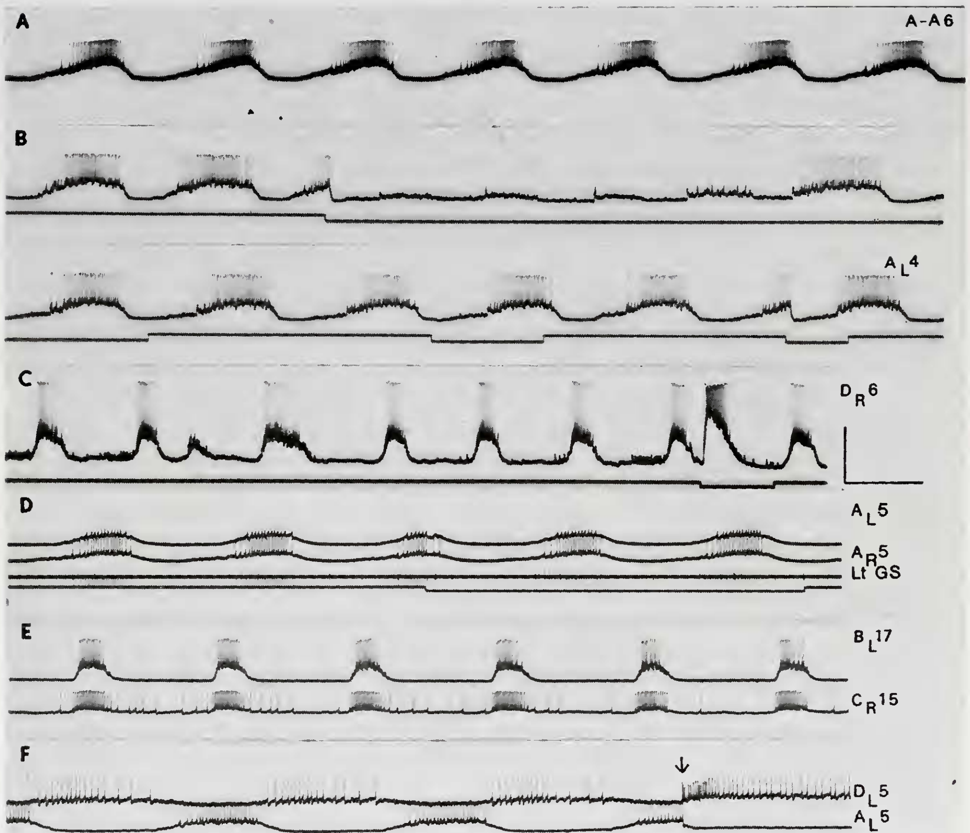

FIGURE 3. Examples of units from *B. cariosus* with regular, patterned output. Grid reference locations noted with each record. A, B. Characteristic Midline Inhibited Burster (MIB). In B, note the effect of "light-off" (lower trace). If off occurs during a burst, that burst is inhibited and subsequent bursts reset. If off occurs during the silent phase there is no significant effect, and "on" causes no change whenever it occurs. C. Lateral Excited Burster (LEB) showing the effect of "light-off" (lower trace). D, E. Illustrates the fact that MIBs are in phase with other MIBs and LEBs are in phase with LEBs. F. Simultaneous recording from a midline burster (lower trace) and a lateral burster (upper trace) to show reciprocity. Calibration is A, B, D, E, F, vertical, 50 mV, horizontal, 4 seconds; C, 25 mV, 4 seconds. Lt GS represents left great splanchnic. Unlabeled lower traces monitor light; downward deflection is "off." Stimulus was delivered to right great splanchnic at arrow in F.

burst than the MIBs (compare Fig. 3B, C, and see Fig. 3E). Membrane potentials are difficult to determine with certainty because of the frequent oscillations. In cells that become silent, however, LEBs have higher membrane potentials (40–50 mV) than MIBs (30–40 mV), but there is some overlap. Action potential amplitude is variable, but does not overshoot zero potential.

The duration of inhibition or excitation depends upon the immediate history of the preparation. If a number of decreased illumination events have occurred the effect on the unit appears to be phasic (1–4 sec) ; if a period of adaptation lasting

10 minutes or more has preceded the shadow, the effect is long-lasting (>15 sec). In short, accommodation is seen, as described (Gwilliam, 1965) for the shadow reflex. This corresponds to the behavior of the intact animal.

An occasional inhibited burster is found laterally, and on one or two occasions a cell that is both inhibited and excited, depending on when in the burst cycle light-off occurs, has been seen. In general, however, the populations are distinct even though there is considerable variability in details of the patterned output each produces.

Temporal characteristics of bursters. The fact that these two kinds of cells respond oppositely to "light-off" suggests that their bursting patterns would be reciprocal. This is indeed the case as shown in Figure 3F. I have never seen two inhibited bursters 180° out of phase, although they may be out by some lesser amount (Fig. 5A). The length of the burst, the number of spikes per burst, and the frequency of the bursts, can be quite variable and are different in the two species. Interburst spikes are frequently seen (Fig. 3E). They may be the result of some slight injury to the cell, or they may be part of the normal pattern (Cf. Burrows, 1974, Fig. 1). Either explanation may be applicable under certain conditions. There is no reason to assume that muscles acting on a hydrostatic skeleton would go to zero tension. In fact, quite the opposite is likely as a means of maintaining turgor.

Correlation with behavior. The burst cycle length in each species falls within the cycle length of fishing and/or pumping behavior in the intact animals as they are observed at comparable temperatures (11°–17° C) in the laboratory (Gwilliam and Bradbury, 1971, for *B. cariosus*). Further, long term recording from a single MIB corresponds to a behavior that is characteristic of barnacles. In intact animals, fishing or related behavior will be initiated, apparently spontaneously, and will also cease for no obvious reason. This behavior occurs at irregular intervals, and the animal may remain closed for considerable periods of time. Mid-line bursters in an isolated CNS "behave" in a manner that reflects the behavior of the intact animal (Fig. 4). This demonstrates the persistent nature of the patterned output of such cells and the lack of dependence on any extrinsic timing cues. No evidence of a circadian rhythm in the behavior has been noted (Cf. Sommer, 1972).

Further indication of the relationship of these cells to activity in the intact animal may be obtained in the following ways. First a moment's reflection suggests that the MIBs are at least temporally related to *extension* of the barnacle (accomplished by muscles acting on the hydrostatic skeleton) because they are inhibited at "off" which would be a necessary event at withdrawal induced by a shadow. The reverse is true of the LEBs, and so they are probably involved in *retraction.* Secondly, it is possible to record from single cells and the adductor muscle simultaneously. As shown previously (Gwilliam and Bradbury, 1971), the adductor muscle junction potentials may also display a rhythm of the same cycle length as the patterned activity. From observations of adductor muscle activity in a pumping barnacle, it can be ascertained that the adductor is relaxed and being extended during body extension, and it contracts during body withdrawal. If one now examines the relationship between a burster and the adductor junction potentials, a conclusion about the function of the bursters may be drawn (Fig. 5B, C). Thirdly, ascertaining the nerve through which the axons of the bursters exit from

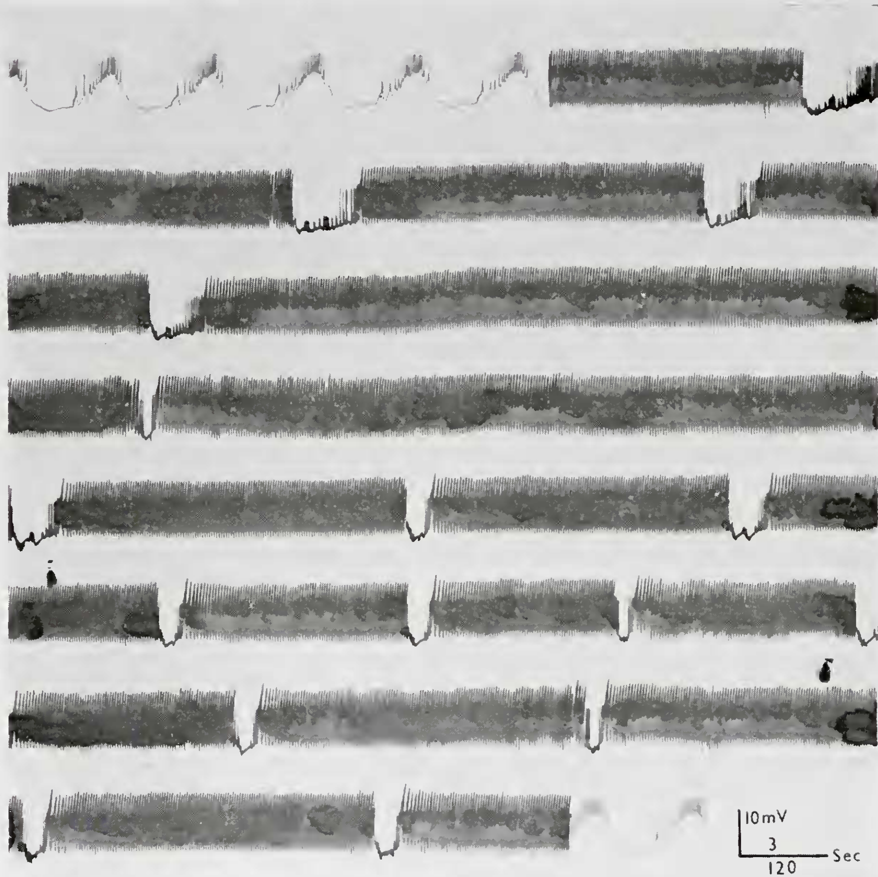

FIGURE 4. Approximately 2 hours and 20 minutes from a continuous record of 20 hours and 25 minutes duration from a Midline Inhibited Burster from *B. cariosus*. This portion is from 8:00 to 10:20 PM. The total run was from 3:15 PM one day to 11:40 AM the next day. Chart speed was increased at beginning and end of record to show characteristic normal burst pattern. Calibration is as noted. Horizontal bar is 3 seconds for beginning and end of record, 120 seconds for remainder.

the ganglion and then tracing the nerve to the muscle it serves can be useful (Fig. 5D; Fig. 6). In cases where the nerve trunk serves many muscles of different function (such as the great splanchnic), this procedure is less useful, but in some cases the nerve distribution is limited and permits the functional conclusion to be drawn. Fourthly, the phase of a burster with the patterned output of various nerves can be useful in some instances. For example, knowing how a particular burst externally recorded relates to the adductor, and knowing how a burster intracellularly recorded relates to the nerve, will permit assigning a role (extension or contraction) to the burster.

Examples of the last three kinds of evidence are shown in Figure 5, B–F, and

bear out the conclusion based on the response to shadows. The fact that some of these cells have a demonstrable 1:1 coincidence with a fiber in a particular nerve and can be antidromically stimulated *via* the nerve is good evidence that the nerve recorded from contains the axon of the penetrated unit, and supports the idea that the bursters are motor cells. One unit illustrated (Fig. 5D) is a lateral burster,

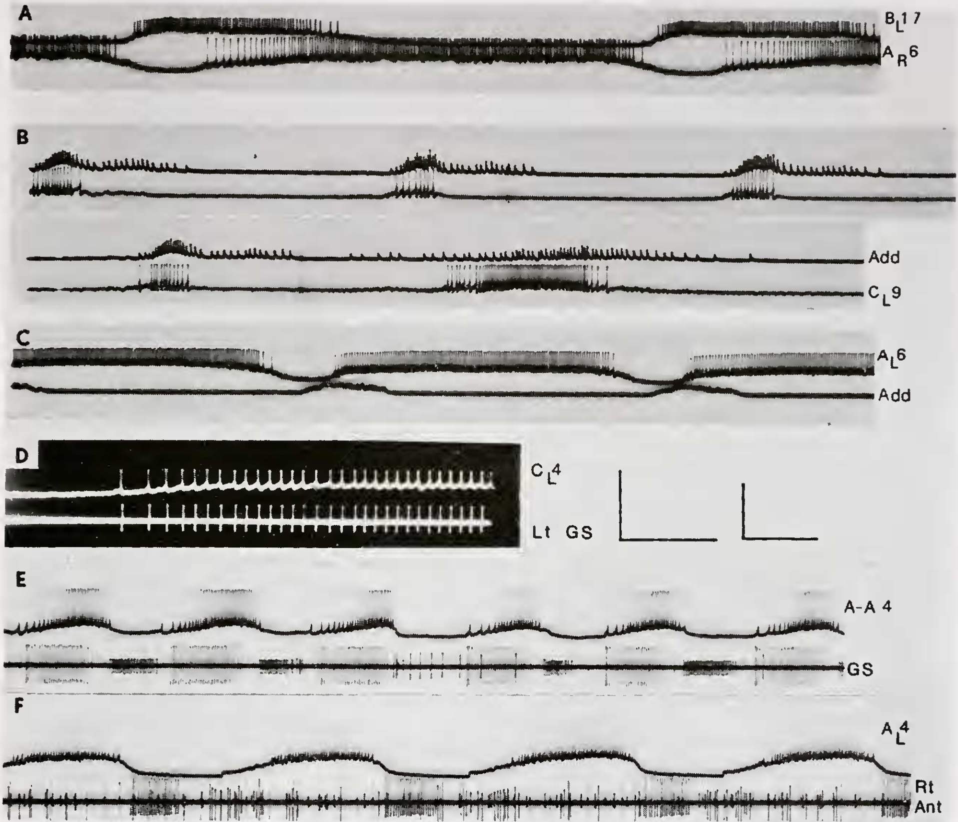

FIGURE 5. Temporal relationships: A–D, *B. hameri*; E. F, *B. cariosus*. A. Simultaneous recording from two inhibited bursters, one well caudad of the other (B_L 17, upper trace) showing overlapping patterns that would be expected of a posteriorly originating, anteriorly progressing wave of muscle contraction driven by these neurons. B. Simultaneous recording from an adductor muscle fiber (upper trace) and a lateral burster. Note that they are in phase as would be predicted for units activated at "off" that function in the withdrawal-closure response. C. The same for a midline burster (upper trace) and the adductor muscle, these two being out of phase. D. Intracellular recording from a lateral burster (upper trace) and an external recording from a branch of the ipsilateral great splanchnic nerve. The 1:1 correspondence indicates that the axon of C_L4 is in that branch of the great splanchnic. E. Intracellular recording from a midline burster (upper trace) and an external recording from the great splanchnic. Note that of the two obvious bursters in the great splanchnic, one is in phase, the other out of phase with A–A4. F. Similar type of recording with the right antennular nerve. Calibration is A–D, left calibration figure. In A and C, vertical bar is 60 mV, horizontal, 4 seconds; B, 7.5 mV (upper trace), 75 mV (lower trace), 4 seconds; D, 60 mV, 2 seconds. The right calibration figure applies to E and F. The vertical bar represents 5 mV; horizontal bar, 2 seconds.

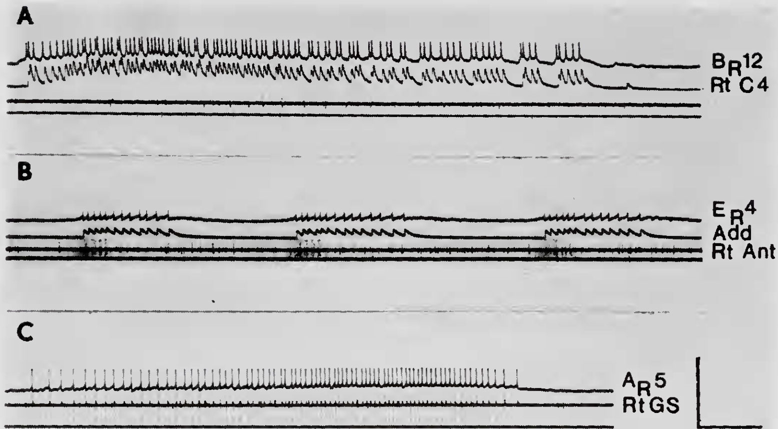

FIGURE 6. *B. cariosus.* A. Posteriorly located lateral burster (upper trace) and the muscle it drives at the base of the left fourth cirrus. B. Anteriorly located lateral burster (upper trace) and the adductor muscle fiber it innervates. C. A midline burster (upper trace) with the branch of the great splanchnic containing its axon. Rt Ant represents right antennular nerve; Rt GS, right great splanchnic nerve. Unlabeled bottom traces monitor light. Calibration is 50 mV, 0.5 seconds.

and its axon apparently exists through a branch of the great splanchnic nerve that innervates the adductor muscle. Similar records have been obtained from the midline bursters, the mid-dorsal nerve, and a branch of the great splanchnic nerve which can be traced to muscles important in the hydrostatic extension of the trunk and cirri (Fig. 6C).

Final proof that certain of the cells described as "bursters" are in fact motor cells has been obtained in preparations that include three pairs of cirri (those that make up the "fishing net") and/or the scutal adductor muscle. It has been possible to penetrate and identify (by their electrical properties) units in the posterior lateral aspect of the ganglion as LEBs and by passing current fire the cell and observe localized contractions in a single cirral ramus coincident with that firing. On a few occasions penetrations of a burster and the muscle fiber it drives have been accomplished (Fig. 6A, B) both in cirral muscles and in the adductor. Such units are identifiable as lateral excited bursters. It has not been possible to make such positive identification of MIBs, because the hydrostatic muscles that lead to extension are not as easily prepared as the "flexors." One-to-one correspondence between a midline burster and the motor supply to hydrostatic extensor muscles, however, has been obtained (Fig. 6C). This particular cell was successfully injected with procion yellow which confirms the mid-ventral location, the soma recording site, and the axon going in the direction of the great splanchnic nerve.

Origin of the bursting pattern. The next question that arises is one concerning the relationship of the bursters to each other. Cells, such as these, that have reciprocal patterns may well be thought of as being mutually inhibitory. This is not true of these units. Changing the frequency or introducing spikes during the silent period of one burster has no effect on any other burster from which I have been able

to record. Cells other than bursters have been driven as well, and to date no clear influence of any other unit—other than the photoreceptor—has been discovered. Gross stimulation of cirral nerves and the great splanchnic nerve results in the same inhibition-excitation phenomenon seen when a shadow is cast, but those are the only influences seen to date (Fig. 3F). Attempts to start a silent burster by stimulation of nerve trunks have not been successful. It seems clear that whatever controls the bursting pattern exerts its influence on each burster and does not rely on these cells influencing each other, *i.e.*, there is no evidence that the bursters are coupled in any

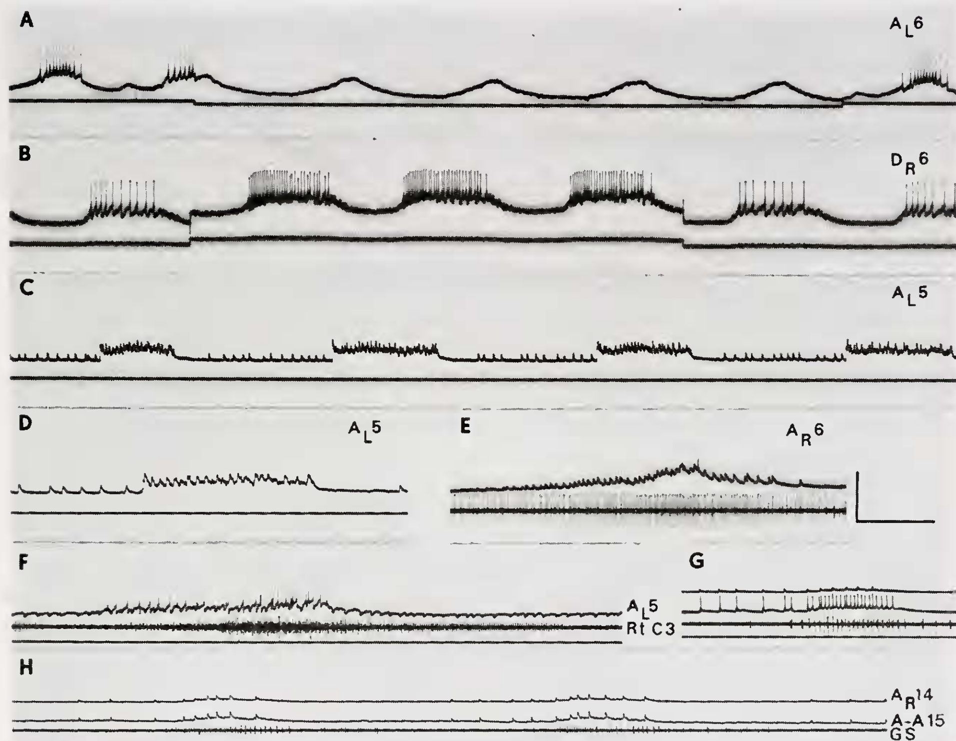

FIGURE 7. *B. cariosus:* A. Midline burster subjected to hyperpolarizing current (monitored on lower trace) sufficient to completely block spiking. Note occurrence of rhythmical membrane depolarization without significant re-setting. B. Depolarizing current applied to a lateral burster, again showing no effect on burst period. C. Midline burster showing post-synaptic potentials in the absence of spiking that frequently occurs spontaneously. EPSPs are prominent, and some indication of IPSPs may be seen in D, a record from the same cell at higher film speed. E. A midline burster subjected to hyperpolarizing current clearly showing EPSPs. F. Midline burster at higher gain to illustrate IPSPs especially during interburst interval. G. Simultaneous recording from neighboring cirral motoneurons (classed as LEBs) to illustrate the mechanism of synchronization. A_R 14 is spontaneously nonspiking and shows some EPSP coincidence with spikes in A–A15. H. A–A15 was hyperpolarized to suppress spiking and now shows 1:1 EPSP coincidence implicating a single presynaptic unit driving both cells. Rt C3 represents right 3rd cirral nerve; GS, great splanchnic. Calibration is A-C, E, G, 40 mV, 2 seconds; D. H., 40 mV, 0.8 seconds; F, 14 mV, 2 seconds.

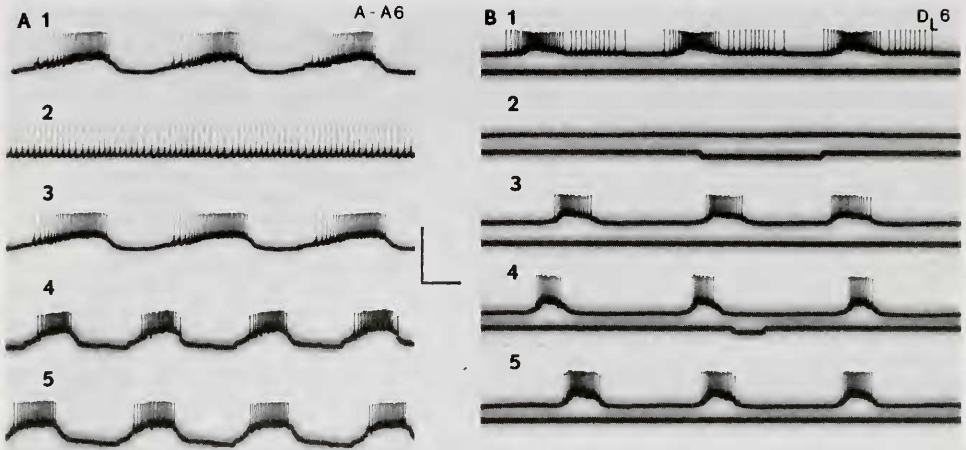

FIGURE 8. *B. cariosus:* Origin of patterning in bursters. A 1–5, Midline Inhibited Burster; B 1–5, Lateral Excited Burster. A 1. Spontaneous pattern in Instant Ocean artificial sea water. A 2. 15 minutes after changing to high magnesium, low calcium medium. A 3. 15 minutes after return to Instant Ocean. A 4. Several hours later, pictures taken 15 minutes after change to simple artificial sea water. A 5. 15 minutes after change to low sodium control medium. B 1. Spontaneous pattern in Instant Ocean. B 2. Two minutes after changing to high magnesium, low calcium medium. B 3. Twenty two minutes after return to Instant Ocean. B 4. Two minutes after changing to low sodium control medium. B 5. Two minutes after return to Instant Ocean. Calibration is vertical, 50 mV, horizontal, 2 seconds. *NOTE:* The change in burst frequency and form as seen between A 3 and A 4, A 5 and between B 1 and B 3, B 5 is not uncommon over a period of time. Such changes cannot be ascribed to experimental manipulations.

direct way (Mulloney and Selverston, 1974a, b; Selverston and Mulloney, 1974; Hoyle, 1964).

Examination of records of bursting patterns under various conditions demonstrates that there is a great deal of synaptic input that serves either to modulate an endogenous pattern, or to create the pattern, or both. On occasion, spontaneous depolarization occurs that does not lead to spiking. Such records sometimes show depolarizing PSPs on the rising phase and hyperpolarizing PSPs on the falling phase. This phenomenon suggests that the burst is driven, but it is also possible that these are simply modulating inputs.

In order to determine if the bursts are endogenously generated, two tests were performed: a) injecting depolarizing and hyperpolarizing current and observing the effect on burst patterns; and b) using high Mg^{2+}, low Ca^{2+} perfusing medium to block transmitter release and observing the effect on bursts.

Examination of records of impaled bursters when they are not spiking often shows trains of EPSPs that occur at bursting frequency (Fig. 7C, D). Hyperpolarizing the cell will suppress spiking and often permit these post-synatic potentials to be seen (Fig. 7A, E). Membrane oscillations occur at the expected interval, *i.e.*, no re-setting occurs. This is also true if the cell is depolarized. The latter leads to higher frequency firing and slightly longer bursts, but the periodicity of the bursts remains essentially unchanged (Fig. 7B). These facts suggest that the burst is driven by synaptic input.

Further evidence to support this comes from observing the effect of changing the medium to one containing high magnesium and low calcium which is known to block transmitter release in other systems (Rubin, 1970; Gainer, 1972). While it is difficult to hold cells long enough to accomplish a complete change of the bathing medium to the high Mg^{2+}, low Ca^{2+} solution and then return it to artificial sea water, some successful manipulations have been carried out. Under the experimental medium, all signs of the bursting pattern disappear and the baseline becomes smooth, indicating no synaptic input. In some MIB cells, the cell begins spiking at a regular frequency. In either case the bursting pattern returns when the medium is changed back to sea water (Fig. 8A). Some MIB cells and all of the LEB cells successfully tested simply become silent under high Mg^{2+}, low Ca^{2+} treatment (Fig. 8B).

Synchronization. Observations of cirral contractions show that most of the muscles of a cirrus contract simultaneously. This means that the motoneurons driving the muscles must be synchronized in some fashion, and the fact that members of a given class of bursters are in phase is indicative of some synchronization mechanism. The most convenient way to examine this is to impale LEBs close together in the posterior part of the ganglion where it has been shown LEBs are cirral motoneurons. Cobalt back-filling *via* a cirral nerve establishes that cell bodies with axons in that nerve are clustered around the root. By impaling cells lying physically very close together at the root, it is sometimes possible to demonstrate that they have a common presynaptic drive unit (Fig. 7G, H) that serves to synchronize the motoneurons.

Thus, it seems clear that the patterned output of the bursters is driven by input from other cells. One possibility is that there is a minimum of two presynaptic cells, both excitatory to the bursters, and mutually inhibitory. Such a system (two presynaptic units) is required because of the opposite actions induced by a shadow and normal pattern reciprocity. A single unit, having both excitatory and inhibitory actions on *two* follower cells, however, has been reported in *Aplysia* (Kandel, Frazier and Coggeshall, 1967), so it is not unreasonable to postulate a similar mechanism here for the presumed second-order cells in the shadow reflex pathway (Millecchia and Gwilliam, 1972).

There is, however, one clear effect on the bursters, and that is the inhibition-excitation that occurs at "light-off." Suitable records illustrate that inhibition takes place on a presynaptic unit, because this event *does* reset the burst pattern (Fig. 3B) and may act directly on the unit or units that generate the patterning.

As yet no candidates for the "oscillator" have surfaced. No oscillator neurons having the characteristics described by Mendelsohn (1971) or Pearson and Fourtner (1975) have been seen, nor have any units that have a direct affect on burst pattern. It can easily be demonstrated that whatever the burst generating mechanism is, it is not restricted to the supra-esophageal ganglion. Removal of the supra-esophageal ganglion has no effect on patterned output from the ventral CNS once the injury effect has terminated.

Discussion

There is still a great deal that may be learned concerning the generation of patterned output and its relation to behavior in the barnacle preparation. This

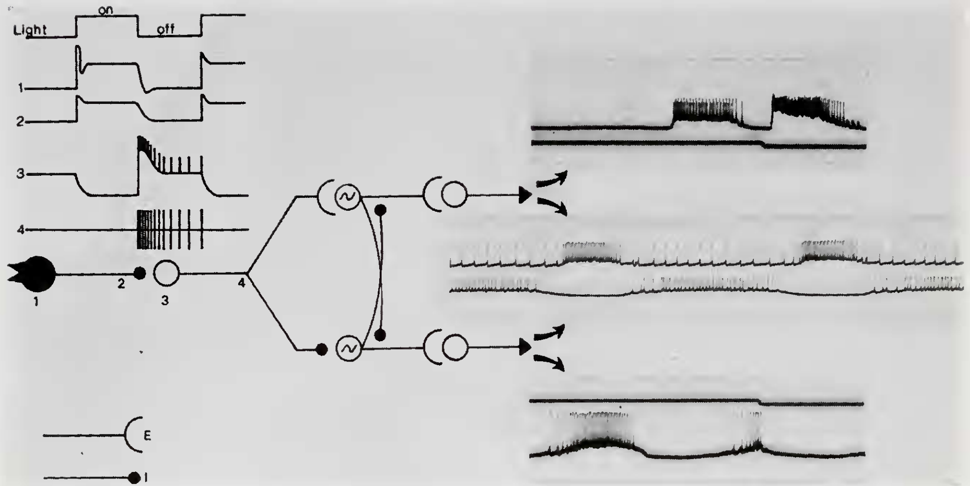

FIGURE 9. Hypothetical circuit to account for reciprocity in the output of the burster cells in *Balanus*. Upper left inset illustrates events at the numbered positions indicated on the diagram. Upper trace indicates light-on (upward deflection) and light-off (from Millecchia and Gwilliam, 1972). 1 shows photoreceptor; 2, photoreceptor axon; 3, second-order cell in supra-esophageal ganglion; 4, fibers in circumesophageal connective. Sine-wave symbol indicates an "oscillator" of unknown type. Upper and lower records illustrate effect of "light-off" (downward deflection of second beam) on LEB and MIB respectively. Middle traces illustrate normal bursting reciprocity. E. represents an excitatory synapse; I an inhibitory synapse.

report is an initial survey and is intended to be primarily descriptive of the neurons, their locations in the ganglion, and some of the properties that will permit characterization of the units. The rigorous demonstration of particular units controlling particular muscles has begun, and the general temporal relationship between the bursters and actions of muscles in extension and retraction has been established.

While the presence of a number of types of cells is recognized, interest has centered on those cells designated as bursters. This is not to imply that other units lack patterned activity, but the same kind of link to behavior as is seen in the bursters has not been established.

If one examines the output of the burster cells, it is not necessary to invoke peripheral inhibition to account for the normal extension-retraction activity. It can be seen from the records that the two types of bursters are reciprocal, that one is inhibited and the other is excited by the shadow. This kind of neuronal activity can account for much of the behavior in the intact animal given the assumption, supported by evidence, that these bursters are indeed motor neurons driving the appropriate muscles.

The output recorded from the bursters is quite variable. Much of the variability in amplitude of electrical events can be explained by invoking electrode tip position in relation to a distant spike generating site and synaptic region. The potential changes seen in the bursters consist of spikes, events best described as IPSPs and EPSPs, and slow oscillatory potential changes. Post-synaptic events without spikes or slow oscillatory potentials are commonly seen (Fig. 7C, D), and slow oscillatory events without obvious post-synaptic potentials and/or spikes are also sometimes

seen. The spikes and post-synaptic potentials have an obvious explanation, but the slow potentials are not as readily explained. The variety of forms the slow changes display suggests they may be sensitive to electrode tip position and are the product of synaptic bombardment. The oscillations cannot be suppressed by passing hyperpolarizing current, but the spikes can. Excess magnesium does suppress the slow oscillations, the post-synaptic potentials, and in many cases the spikes as well. Such cells are still capable of spiking, as can be demonstrated by depolarizing the somata through the recording electrode. All of this leads to the conclusion that the patterned output of these cells is the result of the activity of presynaptic units.

The reciprocity of the patterns of the two types of bursters is also evidently due to presynaptic units. In no case have I been able to demonstrate any direct coupling between any of the bursters as tested by observing the influence of the activity of one cell upon another. One rather striking feature of all multiple site recordings, however, is that in any given preparation the periodicity of bursting patterns recorded from up to four sites is the same at any given time (Gwilliam and Bradbury, 1971, Fig. 3). If arhythmicity or silence occurs (as it does spontaneously quite frequently), it happens throughout the system. These observations suggest that one or a few units or a single "system" paces all of the output. Reciprocity in the output, however, is usually taken to imply a minimum of two units, each with inhibitory connections to the other. A recent model postulates a commonly observed phenomenon, Post Inhibitory Rebound, as the mechanism underlying alternating patterns (Perkel and Mulloney, 1974), but other theoretical possibilities exist (*e.g.*, Dagan, Vernon and Hoyle, 1975).

Such a simple system would account for the output of the bursters except for the appearance of IPSPs during the burst. It is not clear that these IPSPs are phasic and occur only on the falling phase (see Fig. 7F) but if they are, a possible source is direct reciprocal inhibition from the oscillator.

A diagram of a circuit that could account for the burster output and its interruption by a shadow is shown as Figure 9. It ignores all other inputs, which must be numerous, that would account for other modulations of output. The observation that stimulation of the cirral nerves and the great splanchnic nerve will cause the same reciprocal inhibition-excitation in the burster as a shadow is clear evidence that other inputs to the system are operative (see Fig. 3F), although it is not clear whether they act on the oscillators, on other presynaptic units, or on the bursters directly.

The diagram is not meant to imply that the "oscillators" are single units, but only that there is a part of the system that produces reciprocal bursts, that it is physically separate from the cells designated in this paper as bursters, and that it is spontaneous. Nor does this model speak to the mechanism of burst generation. Nonendogenous burster models developed to explain pattern generation in the lobster stomato-gastric ganglion (Warshaw and Hartline, 1974; Mulloney, Budelli and Perkel, 1975) may well be applicable, but there is no hard evidence that permits a choice to be made.

It has been possible in this study to record from reciprocating pairs of neurons, and such recordings do not show any signs of direct inhibitory coupling. Recordings from in-phase pairs also fail to reveal any direct coupling. Such negative evidence, however, cannot be regarded as conclusive (Selverston and Mulloney,

1974). Coupling may exist within functional subsets (*e.g.*, between cirral flexor and its antagonist which may be one or more of the muscles acting on the hydrostatic skeleton at some distant point), and would only be seen as a lucky accident of recording. Recording from in-phase pairs that are located physically close together is a more reliable test of coupling and such recordings demonstrate a common antecedent neuron as the mechanism of synchronization.

The cells described here may be compared to the bursty cells seen in the lobster stomatogastric ganglion (Mulloney and Selverston, 1974a, b; Selverston and Mulloney, 1974) and to the neurons controlling ventillation in the locust abdomen (Burrows, 1974). Some of the cells in the barnacle produce bursts of activity that are remarkably similar to the locust motoneurons both in burst fine structure and timing. The methods of activation also seem to be similar, *i.e.*, the bursters are synaptically driven and/or modulated by antecedent neurons that account for the patterning. While the patterns in these units may resemble those seen in the stomatogastric ganglion, there is no evidence of the extensive electrotonic coupling or indeed any direct synaptic connections as have been established in those motor neurons.

Another question that must be addressed is the numbers of cells referred to as bursters. If they are indeed motor neurons then there should be some correspondence between cell numbers and motor axons. That question has not been seriously considered in this work, partly because details of motor supply to the muscles are not very well known in barnacles. Counts of muscle fibers in a pair of rami from a single cirrus show that the rami together contain a total of about 70 muscle fibers. In addition, there are several fibers in the base, so a reasonable guess would be about 100 muscle fibers per cirrus. Some intracellular records from muscles show dual innervation (*i.e.*, a "small" and "large" junction potential in a single fiber as well as multiple muscle fiber activated by a single motor neuron). The degree of flexion in a cirrus achieved by driving one cirral motor neuron (LEB) would suggest each neuron innervates more than one muscle fiber, but these observations are still in the preliminary stage, and the objectivization of muscle contraction in the cirri is proving technically difficult. The use of a transducer tube (RCA 5734) will probably make this possible and permit direct studies of tension as related to motor cell activity.

The work with *Balanus hameri* was carried out at the Marine Sciences Laboratory, Menai Bridge, Anglesey, North Wales, U. K. I wish to thank Professor D. J. Crisp for permission to work at the laboratory. I especially wish to thank Dr. Derek A. Dorsett, his associates and students, who provided me with equipment, invaluable assistance, and an atmosphere conducive to work. Diolch yn fawr!

It is also a pleasure to acknowledge my gratitude to Ms. Andrea Frost for her able assistance over the past three years.

Summary

The isolated barnacle central nervous system has been used to study neurons related to the shadow reflex and rhythmical behavior. The function of certain of the neurons has been ascertained by exploiting a preparation that included three pairs of cirri and the scutal adductor muscle which permitted simultaneous neuron-

muscle fiber intracellular recording. The inclusion of the median photoreceptor in all of the preparations provided a means of assaying the effect of shadows on the system.

The absence of peripheral structures in the isolated preparation coupled with the observation that rhythmical bursting patterns will persist for hours is strong evidence for one or more spontaneous "oscillators" in the CNS that are capable of driving the basic fishing, pumping behavioral repertoire of the barnacle.

The principle finding reported is that there are two classes of bursters, identifiable by location, bursting pattern, and response to "light-off" that exhibit reciprocity. Evidence is presented to link the laterally located bursters (excited at "light-off") to withdrawal, the mid-line bursters (inhibited at "light-off") to extension, in the intact animal.

Evidence the bursters are driven and/or modulated by synaptic input is provided by observations on nonspiking bursters, by passing hyper- and depolarizing currents, and by treating the ganglion with high Mg^{2+}, low CA^{2+} artificial sea water. In some of the bursters, synchronization is accomplished by a common antecedent interneuron.

Simultaneous neuron-muscle recordings and locating axons of impaled neurons in peripheral nerves has established some of the bursters as motor neurons.

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